



## **Exploring gene expression profiles in germinating Barley.**

Loraine Watson, Timothy A. Holton and Robert J. Henry

CRC for Molecular Plant Breeding, Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW 2480

# **Abstract**

Germination rate plays an important role in determining the malt quality obtained from barley. The current research aims to use microarray analysis to examine gene expression in germinating barley and determine genetic components controlling germination.

Microarray technology has the potential to revolutionise the biological sciences by providing a means of concurrently extracting vast quantities of genetic information from complementary DNA (cDNA) libraries. The technique will be used to compare (a) gene expression levels at different time points during germination; (b) genes expressed within the embryo to those expressed in the aleurone layer and endosperm, and; (c) gene expression between malting and feed quality varieties of barley.

The construction of a suitable cDNA library from 1 to 4 day old barley embryos constitutes an important first step in the microarray analysis process. Two cDNA libraries will be used to provide clones for the microarrays. One library requires a relatively large amount of messenger RNA (mRNA) for construction. The other library, constructed using a new method that utilises the Polymerase Chain Reaction (PCR) to amplify small amounts of cDNA, has the potential to provide very specific clones. The new PCR-based method also raises the possibility of producing cDNA libraries from specific tissue sources - thus enabling a high degree of gene targeting.

# **Introduction**

Barley germination rate, coupled with the timing and extent of starch-degrading enzymatic activity, plays an important role in determining malt quality. However, these malting characteristics can be dramatically influenced by environmental conditions during grain development (Wallwork *et al.* 1998). Therefore, while consistency and homogeneity are desirable malting barley traits, they are rarely attainable without intervention.

Understanding the fundamental biological processes that underpin malt production is a key factor for the future development of improved malting barley varieties. While the biochemical and physiological components of barley germination and malting have been studied for many years (Mikola and Enari 1970; Briggs 1978; Briggs and Cornish-Bowden 1981; Enari and Sopanen 1986; Briggs 1987, 1992; Fincher and Stone 1993), a molecular description of the processes involved remains to be fully explored. To date, molecular research has focussed on either defining specific barley gene functions (Acevedo *et al.* 1996), examining gene expression within specific tissues - especially the aleurone layer (Rogers 1985), or on specific gene expression patterns under the influence of, for example, hormone induction (Chandler and Robertson 1994).

## **A New Era in Genetic Exploration**

One of the major research limitations imposed has been the need to focus on a small number of genes and proteins at any one time. However, recent robotic innovations have begun to revolutionise the biological sciences by providing a means of concurrently examining the entire gene expression profile of an organism or tissue (Schena *et al.* 1995; Schena *et al.* 1998; Brown and Botstein 1999). The new technology, Microarray Analysis, allows a researcher to spot an entire expressed genome (usually a complimentary DNA (cDNA) library) onto a glass slide. The slide can then be probed with fluorescently-labelled single-stranded DNA (ssDNA), synthesised from an organism's messenger RNA (mRNA), to provide a 'snap-shot' of the gene expression pattern at any point in time under any given set of conditions. No longer restricted to examining the expression of single genes, the researcher now has the ability to extract vast quantities of genetic information from these cDNA microarrays (Ramsay 1998). Indeed, so much data can be generated that care must be taken to design appropriate questions and use appropriate data mining techniques in order to extricate meaningful information from the microarrays (Richmond and Somerville 2000).

Given its necessary reliance on robotics, microarray technology is expensive and has, therefore, been utilised mainly by the research areas of human genome exploration and drug discovery (Schena *et al.* 1998). However, the inherent scientific benefits of being able to examine gene interactions and the likelihood of agronomic dividends through fast-tracking the elucidation of molecular pathways has seen the recent implementation of microarray technology in the plant sciences (Lemieux *et al.* 1998). To date, most of the published plant-based microarray research relates to the model plant, *Arabidopsis thaliana* (Ruan *et al.* 1998; Hu *et al.* 1999; Girke *et al.* 2000). However, researchers are also beginning to explore the use of microarrays for understanding gene expression patterns in major agricultural species (Ewing *et al.* 1999; Arimura *et al.* 2000).

## **Exploring Barley Gene Expression**

The Cooperative Research Centre for Molecular Plant Breeding (CRCMPB) has funded a project that will examine the gene expression profiles of germinating barley utilising microarray technology, and should help to determine the genetic components involved in controlling germination. Very little genetic information derived from

studies of intact barley kernels is currently available. Therefore, one of the research project outcomes is to provide a large amount of fundamental data that can be utilised by future researchers in the search for genetic characteristics leading to the development of more efficient malting barley varieties.

To achieve this aim, three approaches will be taken. The first will be to look at gene expression patterns of a specific malting barley variety at different time points during the first four days of germination. The second phase will involve differentiating between expression patterns in the embryo and those of both the aleurone layer and the endosperm. Finally, comparisons will be made between the expression profiles of malting and a non-malting barley variety with a phenotypically different germination profile.

## **Producing a Suitable cDNA Library**

To begin the process, a cDNA library capable of providing answers to all of the questions under investigation must be constructed. Since the period of interest is the portion of germination that is of importance to the malting process, the library should encompass as much genetic information as possible from the first four days after imbibition. The library should also ideally provide an 'average' of the types of genetic information that might be found across the spectrum of malting barley varieties.

A further consideration is the future possibility of being able to construct a library from a single barley embryo. Eliminating any dilution of specific gene expression patterns caused by the 'averaging-out' that necessarily occurs in libraries constructed from a number of embryos would be a major advantage in a single-embryo approach. Therefore, testing the feasibility of constructing such a library, and assessing its scientific usefulness, is also considered to be an important project goal. Using the microarray technique introduces the possibility of making direct comparisons between libraries constructed by different methods with different initial tissue volumes by spotting the two libraries side by side and monitoring any anomalies in hybridisation levels. Hence, two libraries could be constructed.

# **Materials and Methods**

## **Choosing a barley library candidate**

Germination experiments were carried out on a number of barley varieties to determine (a) mean embryo weight; (b) mean germination rate; (c) whether an 'average' phenotype for different growth stages could be observed; and (d) which variety would be best suited to the task of producing the cDNA libraries. The malting varieties tested were: Tallon, Schooner, Grimett, Stirling, Franklin, Arapiles, Alexis, Sloop, Harrington and Chebec. The feed quality barley, Galleon, was chosen as the non-malting variety. Kernels were sterilised by rinsing in 100% ethanol followed by soaking in <50% bleach solution for 20 min, then washed twice with MilliQ water. Sterilised grains were then transferred to petri dishes containing sterile Whatmann filter paper and 5ml MilliQ water. Closed petri dishes were sealed into foil envelopes

to exclude light and incubated at 17°C for periods of 24hr, 48hr, 72hr and 96hrs respectively. After the allotted time period had elapsed, 8 embryos were harvested individually using tweezers and an Olympus dissecting microscope and immediately immersed in liquid nitrogen. Before harvesting, the number of germinated kernels was counted and an average phenotype for each time period was recorded. The mean germination scores for each barley variety were determined and, along with features such as ease of handling, used to select the library source. The data was also used to select a number of other varieties that could be used for comparative microarray analysis at a later stage.

## RNA extraction

Two different methods of mRNA extraction were used. For RNA extraction using small quantities of tissue (100mg), the plant tissue protocol from Qiagen's RNeasy<sup>®</sup> Mini Extraction Kit was followed. For large-scale RNA extractions where a greater amount of tissue was required, a modified CTAB/Lithium Chloride extraction protocol, followed by the RNA cleanup protocol from Qiagen's RNeasy<sup>®</sup> Midi Extraction Kit was utilised.

## Library construction

Two Stratagene kits have been chosen for producing the cDNA libraries. The Stratagene ZAP-cDNA<sup>®</sup> Synthesis Kit utilises a ZAP-cDNA<sup>®</sup> Gigapack<sup>®</sup> III Gold Cloning Kit and requires between 3 - 5µg poly(A)<sup>+</sup> RNA. If we assume that 1-2% of total RNA is poly(A)<sup>+</sup> RNA, the amount of mRNA (or total RNA) required is approximately 250µg. Conversely, a more recent Stratagene kit - the PCR cDNA Library Construction Kit - is optimised for 100ng total RNA (Neiditch *et al.* 1999). Data on average total RNA yields (not shown) suggests that the PCR-based method would be a feasible option for constructing a single-embryo library.

The use of PCR amplification has the potential to produce a library that is biased towards more highly expressed genes since these could be preferentially copied during the amplification process. By constructing the two libraries using the same mixed RNA source (that is, equal amounts of total RNA from each of the four harvest periods) it should be possible to compare library diversity and determine whether the PCR-based method provides adequate scope for gene expression profile analysis. Library construction using 'Alexis' as the library source tissue is currently under way. The ability to target gene expression patterns in single embryos could open new doors to plant molecular genetics, allowing the researcher and breeder to harness individual grain characteristics and more accurately determine the genetic basis for desirable malting qualities.

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